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Article

Epigenetic Inactivation and Subsequent Heterochromatinization of a Centromere Stabilize Dicentric Chromosomes

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Summary

Background: The kinetochore is a multiprotein complex that forms on a chromosomal locus designated as the centromere, which links the chromosome to the spindle during mitosis and meiosis. Most eukaryotes, with the exception of holocentric species, have a single distinct centromere per chromosome, and the presence of multiple centromeres on a single chromosome is predicted to cause breakage and/or loss of that chromosome. However, some stably maintained non-Robertsonian translocated chromosomes have been reported, suggesting that the excessive centromeres are inactivated by an as yet undetermined mechanism.

Results: We have developed systems to generate dicentric chromosomes containing two centromeres by fusing two chromosomes in fission yeast. Although the majority of cells harboring the artificial dicentric chromosome are arrested with elongated cell morphology in a manner dependent on the DNA structure checkpoint genes, a portion of the cells survive by converting the dicentric chromosome into a stable functional monocentric chromosome; either centromere was inactivated epigenetically or by DNA rearrangement. Mutations compromising kinetochore formation increased the frequency of epigenetic centromere inactivation. The inactivated centromere is occupied by heterochromatin and frequently reactivated in heterochromatin- or histone deacetylase-deficient mutants.

Conclusions: Chromosomes with multiple centromeres are stabilized by epigenetic centromere inactivation, which is initiated by kinetochore disassembly. Consequent heterochromatinization and histone deacetylation expanding from pericentric repeats to the central domain prevent reactivation of the inactivated centromere.

Introduction

The kinetochore is a multiprotein complex that mediates spindle-chromosome attachment during mitosis and meiosis and therefore plays a pivotal role in faithful chromosome segregation. The kinetochore is formed on a specific chromosomal locus designated as the centromere. The presence of multiple centromeres on a single chromosome is predicted to cause abnormal spindle-chromosome interaction resulting in mitotic chromosome instability [1, 2]. In most eukaryotes, with the exception of holocentric species, each chromosome contains a single distinct centromere. However, recent findings regarding kinetochore formation on a noncentromere locus, known as a "neocentromere" [3–5], have suggested that a single chromosome contains multiple latent centromeres. Furthermore, several cases of stably maintained non-Robertsonian dicentric chromosomes, possessing two distinct centromeres, have been reported [6]. These studies suggested that the number of active centromeres on which the kinetochore may form is restricted by an as yet undetermined mechanism.

In many model organisms, the centromere is located at the primary constriction site of the chromosome where kilobase- to megabase-sized arrays of repetitive DNA sequences are often found [7]. Recent progress in genomic sequencing in various organisms indicated that the centromeric DNA sequences are widely divergent among species. Furthermore, some neocentromeres do not contain any DNA sequences related to the authentic centromeric DNA repeat [3–5], suggesting that the centromere locus is not determined solely by the DNA sequence; i.e., epigenetic mechanisms may also be important for centromere determination [7]. In contrast to the diversity of the centromeric DNA sequence, many proteins composing the kinetochore are evolutionarily conserved. Among them, CENP-A protein, which is the conserved centromere-specific variant of histone H3, serves as a good hallmark of the active kinetochore; it is found only in a functional kinetochore, regardless of whether such a kinetochore is formed on the authentic centromere or a neocentromere [8, 9].

The fission yeast *Schizosaccharomyces pombe* possesses three chromosomes. The centromere of each chromosome consists of repetitive sequence elements divided into two domains: the 10 to 15 kb central domain on which evolutionarily conserved kinetochore components bind (*cnt* and *imr* elements) and the flanking 10 to 60 kb heterochromatinized *otr* domain composed of *dg* and *dh* elements [10, 11] (see Figure S2A available online). These characteristics of the fission yeast centromere resemble those of higher eukaryotes, and it may therefore serve as a good model system for studies of centromere function and regulation.

In this study, we explored how the artificially generated dicentric chromosome is stabilized in fission yeast. Our findings suggested that the dicentric chromosome causes cell-cycle arrest in interphase, during which one of the centromeres on the dicentric chromosome is inactivated in an epigenetic manner (i.e., without alterations in the DNA sequence). Epigenetic centromere inactivation is triggered by dissociation of kinetochore components and is followed by heterochromatinization, which prevents reactivation of the inactivated centromere. We propose a mechanism of centromere inactivation, which is important for restricting the number of the centromeres per chromosome through centromere inactivation.

Results

Dicentric Chromosomes Induce Cell-Cycle Arrest in Interphase in a Manner Dependent on DNA Structure Checkpoints

To confirm that the presence of multiple centromeres on a single chromosome causes mitotic abnormalities, we

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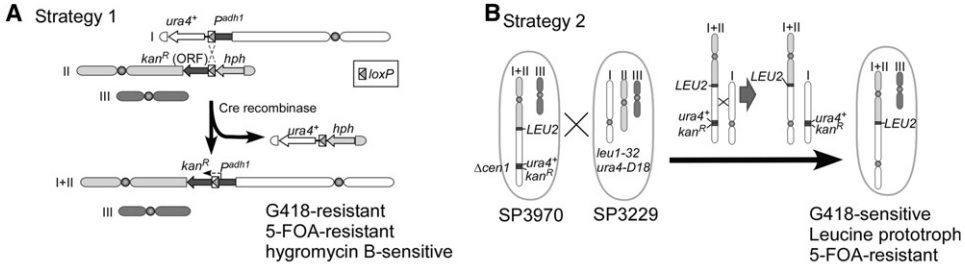


C

time after Cre transformation (h)	frequency of cells longer than 13 μm (%)	septation index (%)
24	32	4
30	40	4
36	54	8
48	50	2
control (GFP only)	0	11

E

	frequency of cells longer than 13 μm (%)	septation index (%)
Δ*rad3*	12	10
Δ*chk1*	12	12
Δ*cds1*	46	12
Δ*mad2*	50	2

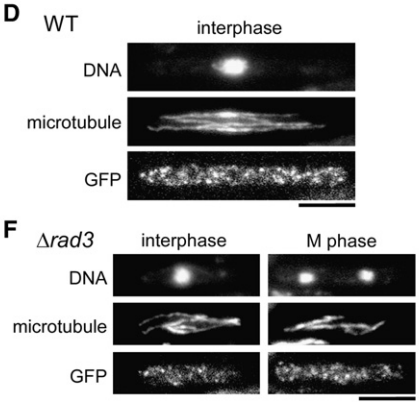


Figure 1. The Dicentric Chromosome Causes Cell-Cycle Arrest in Interphase

(A) Scheme of strategy 1 using site-specific recombination at *loxP* sites by Cre recombinase. Cre-mediated homologous recombination at the *loxP* sites introduced at the ends of chromosomes I and II produced the fused dicentric chromosome.

(B) Scheme of strategy 2 using meiotic recombination. The SP3970 strain has a long chromosome generated by telomeric fusion of chromosome II and centromere-disrupted chromosome I. Meiotic crossover between the fused chromosome of SP3970 and chromosome I of WT produces the dicentric fused chromosome.

(C) The septation index and the length of cells bearing the dicentric chromosome (SP4387) and the control (SP4390) were measured. Fifty SP4387 cells expressing GFP were examined at 24, 30, 36, and 48 hr after introduction of the pREP81-NLS-Cre plasmid, and the proportion of cells longer than 13 μm was calculated. In the SP4390 strain, the GFP gene fused with *adh1* promoter and the *loxP* sequence was integrated at the *lys1*$^+$ locus, so that GFP protein could be expressed constitutively without the pREP81-NLS-Cre plasmid. One hundred and fifty SP4390 cells at exponential growth phase were examined as controls.

(D) Images of an interphase-arrested GFP-expressing SP4387 cells are shown. DNA was stained with DAPI, whereas microtubules and GFP were stained by indirect immunofluorescence. Scale bar represents 5 μm.

(E) The septation index and the cell length were measured in checkpoint-deficient Δ*rad3*, Δ*chk1*, Δ*cds1*, and Δ*mad2* cells bearing the dicentric chromosome. In each mutant, 50 GFP-expressing cells were examined at 48 hr after introduction of the pREP81-NLS-Cre plasmid, and the proportion of cells longer than 13 μm was calculated.

(F) Images of Δ*rad3* cells harboring the dicentric chromosome cells are shown. A representative interphase cell is presented in left panels, while a mitotic cell is shown in right panels. Scale bar represents 5 μm (see also Figure S1).

developed methods to generate dicentric chromosomes in fission yeast cells by fusing two distinct chromosomes. Two different strategies were employed for chromosome fusion: site-specific recombination (strategy 1) and meiotic recombination (strategy 2) (Figures 1A and 1B). In strategy 1, *loxP* sites introduced at the left end of chromosome I and the right end of chromosome II were recombined by Cre recombinase to generate a fused dicentric chromosome consisting of chromosomes I and II (Figure 1A). On the other hand, in strategy 2, the wild-type (WT) strain (SP3229) was crossed with a strain (SP3970) in which chromosome II is fused with chromosome I, in which the centromere was replaced by a G418 resistance marker, *kan*^{R}; meiotic crossing over at the left arm of chromosome I created a fused dicentric chromosome (Figure 1B). In both strategies, cells harboring the dicentric chromosome could be selected by using specific combinations of selective markers.

As predicted, the majority of the cells with the dicentric chromosome were nonviable; the viabilities of the cells were 0.20% ± 0.02% and 0.75% ± 0.13% in strategies 1 and 2, respectively. To examine how the cells were killed by the presence of the dicentric chromosome, we examined the cell morphology by light microscopy. To select cells with the dicentric chromosome under the microscope, the green fluorescent protein (GFP) marker gene was split into two fragments that were placed next to the *loxP* sites in strategy 1 so that recombination between *loxP* sites would regenerate a functional GFP marker gene (SP4387). In GFP-expressing cells (i.e., cells with a dicentric chromosome), the morphologies of nuclei and microtubules as well as the cell length were examined at 12, 24, 30, 36, and 48 hr after introduction of a plasmid containing Cre recombinase under the control of the thiamine-regulated *nmt1* promoter (pREP81-NLS-Cre). GFP-expressing cells were rarely seen at 12 hr and began to be found at 24 hr, consistent with the time course of expression from the *nmt1* promoter, which occurs 10–16 hr after removal of thiamine [12]. Interestingly, cells showing mitotic abnormalities, such as the "*cut*" phenotype [13], did not accumulate significantly at any time point. Instead, the cells showed an elongated cell morphology; the number of cells longer than 13 μm, which were never found among the controls, gradually increased and reached a maximum of 54% at

36 hr (Figure 1C; Figure S1A). Most of these elongated cells possessed single undivided nuclei and the cytoplasmic microtubule array (Figure 1D; Figure S1B). Their septation index dropped to 2% at 48 hr, whereas that in the controls was 11% (Figure 1C). These observations suggested that the presence of the dicentric chromosome caused cell-cycle arrest in interphase, rather than catastrophic chromosomal breakage-fusion-bridge cycle [1].

Next, we examined whether cell-cycle arrest induced by the dicentric chromosome depends on the cell-cycle checkpoint pathways. For this purpose, the length and septation index of the cells with the dicentric chromosome were measured in strains lacking the *rad3*$^+$, *chk1*$^+$, *cds1*$^+$, or *mad2*$^+$ gene (Δ*rad3*, Δ*chk1*, Δ*cds1*, and Δ*mad2*, respectively) (Figure 1E; Figures S1C and S1D). Fission yeast Chk1 and Cds1 are protein kinases involved in DNA damage and replication checkpoint pathways, which prevent the onset of mitosis responding to damage or incomplete replication of the chromosomal DNA, respectively [14, 15]. Rad3 is a PI3K-like kinase required for both pathways [16]. On the other hand, Mad2 is essential for the mitotic spindle assembly checkpoint, which blocks metaphase to anaphase transition until all the chromosomes have bound properly to the spindle [17]. The results of measurement at 48 hr after introduction of the pREP81-NLS-Cre plasmid are summarized in Figure 1E. The proportion of elongated cells (>13 μm) decreased significantly in Δ*rad3* and Δ*chk1*, whereas the septation index was as high as in the control without the dicentric chromosome, indicating that these checkpoint genes are indispensable for cell-cycle arrest induced by the dicentric chromosome. Although the proportion of elongated cells in Δ*cds1* was similar to that in the WT strain, the septation index was elevated to the same levels as in Δ*rad3* and Δ*chk1*, suggesting that Cds1 is also important for maintaining permanent cell-cycle arrest. In contrast, the spindle assembly checkpoint appeared dispensable for this arrest; in Δ*mad2* cells harboring the dicentric chromosome, both the proportion of elongated cells and the septation index were identical to those in the WT strain. These results indicated that the DNA damage and replication checkpoint pathways, but not the spindle checkpoint, prevent cell-cycle progression in interphase in the presence of a dicentric chromosome. One of simple hypotheses explaining these results is that aberrant mitotic segregation of the dicentric chromosome would generate broken DNA ends, which may activate the DNA damage checkpoint causing cell-cycle arrest. However, this hypothesis may not be fully consistent with the finding that, although the Δ*rad3*, Δ*chk1*, and Δ*cds1* cells continued to divide in the presence of the dicentric chromosome, only a small portion of these cells (3/50, 3/50, and 3/50 in Δ*rad3*, Δ*chk1*, and Δ*cds1*, respectively, at 48 hr) showed abnormal mitotic phenotypes potentially causing chromosome breaks (Figure 1F). Another speculative hypothesis is that these checkpoints may also respond to abnormalities in the chromosome structure, such as the presence of multiple kinetochores, and block the onset of mitosis to prevent mitotic loss or breakage of the dicentric chromosome. Further studies are required to determine the mechanism underlying this cell-cycle arrest induced by the dicentric chromosome.

Dicentric Chromosomes Are Stabilized without Alteration of Either Centromeric DNA Sequence

Although the majority of cells with the dicentric chromosome ceased to divide, as discussed above, a small portion of cells survived with stable maintenance of the dicentric chromosome. To gain insight into the mechanism underlying the stabilization of the dicentric chromosome, we analyzed the chromosome structures of the survivors by pulsed-field gel electrophoresis (PFGE) (Figures 2A–2C; Figure S2F). Because essentially the same results were obtained for survivors with both strategies, the results from representative survivors with strategy 2 are shown. Based on the electrophoretic patterns, the survivors were classified into three types: type-A, -B, and -C. The type-C survivors possessed three chromosomes (Figure 2A), suggesting that the dicentric fused chromosome had broken down to two chromosomes, which were healed by de novo telomere addition at the ends (Figure S2E). In contrast, the type-A and type-B survivors possessed only two chromosomes, the larger of which was expected to be the fused chromosome containing two centromeres, i.e., centromeres 1 and 2 (Figure 2A; Figures S2C and S2D).

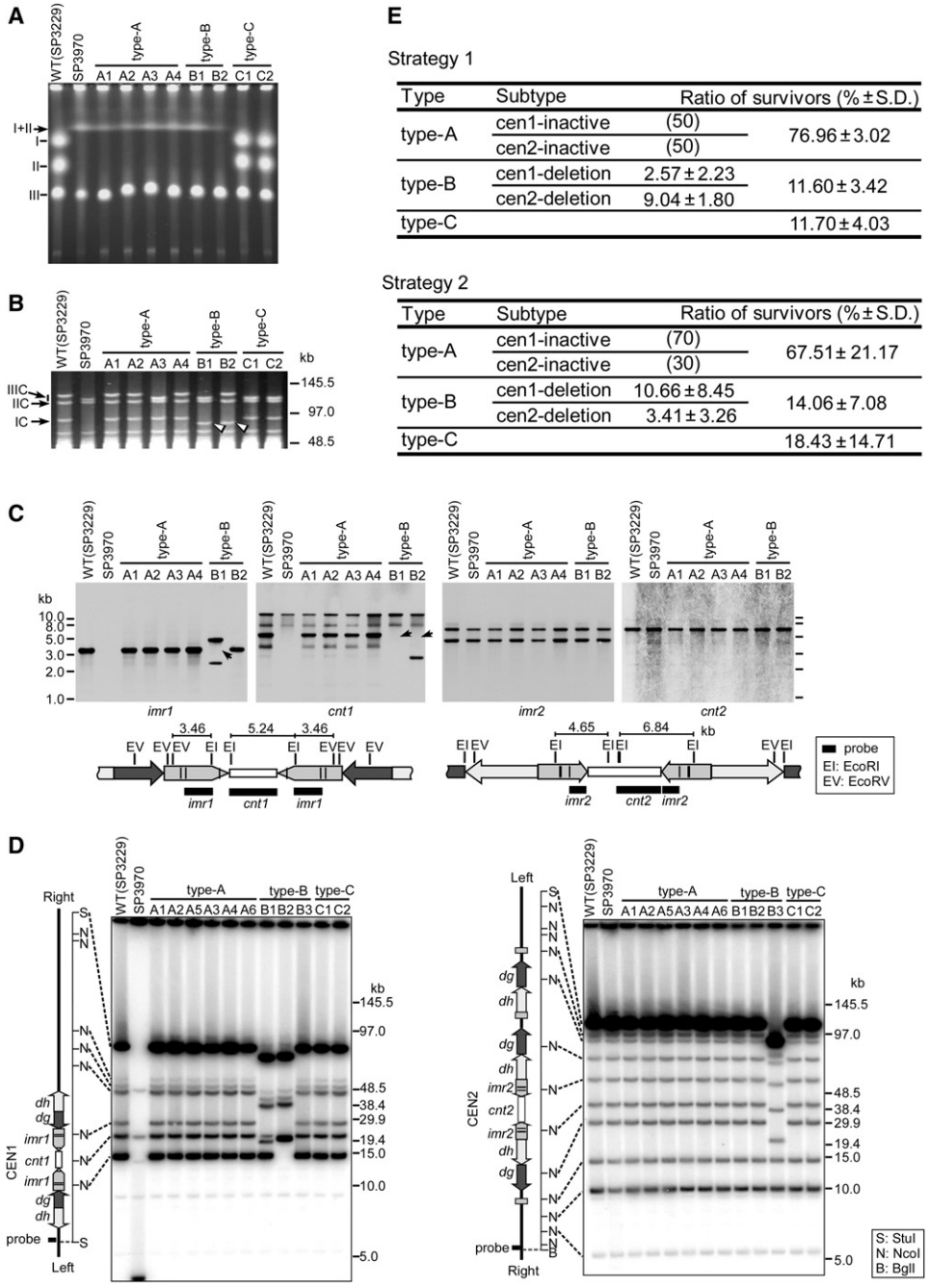
PFGE analysis following restriction enzyme double digestion (StuI and BglI) indicated that one of the centromeres on the fused chromosome was deleted by DNA rearrangement in type-B survivors; either the restriction fragment containing centromere 1 or 2 (IC or IIC) ran faster than those in WT (Figure 2B arrowheads; Figures S2A and S2B). Consistent with this suggestion, genomic Southern blotting analysis showed that a substantial portion of the central domain of centromere 1 (B1 and B2 strains in Figure 2C) or of centromere 2 (B3 strain in Figure 2D and data not shown) was eliminated in type-B survivors. Physical elimination of one centromere probably converts the fused dicentric chromosome into a monocentric chromosome that is stably maintained in type-B survivors.

In the remaining type-A survivors, no obvious DNA rearrangement was detected at either centromere, suggesting that the type-A survivors stably maintained the dicentric chromosome in which the DNA sequences of the centromeres were intact. To confirm the integrity of the centromeres, we mapped restriction enzyme sites (NcoI) in the regions of centromeres 1 and 2 (Figure 2D); as expected, the positions of NcoI sites around centromeres 1 and 2 in type-A survivors were identical to those in the WT. This result suggested that the dicentric chromosome was stabilized epigenetically in the type-A survivors. As discussed below, the dicentric chromosome, on which either of the centromeres lost its function without accompanying alterations in the DNA sequence, probably behaves as a stable monocentric chromosome in the type-A survivors.

The proportions of the three types of survivor in strategies 1 and 2 are summarized in Figure 2E; more than two thirds of the survivors were type-A in both strategies, indicating that the dicentric chromosome was stabilized mostly in an epigenetic manner.

The Kinetochore Is Formed on Only One of the Centromeres on the Dicentric Chromosome in Type-A Survivors

We assumed that one of the centromeres was inactivated on the dicentric chromosome without accompanying alterations in the DNA sequence in type-A survivors and did not function as a platform for kinetochore formation. To test this possibility, we performed chromatin immunoprecipitation (ChIP) analysis to examine whether CENP-A protein (named Cnp1 in fission yeast), which forms the platform for kinetochore assembly [9], bound to the centromeres of the dicentric chromosome in type-A survivors. In WT cells, CENP-A was recruited into the central domains of all three centromeres [18] (Figure 3A,



E

Strategy 1

Type	Subtype		Ratio of survivors (% ± S.D.)
type-A	cen1-inactive	(50)	76.96 ± 3.02
	cen2-inactive	(50)	
type-B	cen1-deletion	2.57 ± 2.23	11.60 ± 3.42
	cen2-deletion	9.04 ± 1.80	
type-C			11.70 ± 4.03

Strategy 2

Type	Subtype		Ratio of survivors (% ± S.D.)
type-A	cen1-inactive	(70)	67.51 ± 21.17
	cen2-inactive	(30)	
type-B	cen1-deletion	10.66 ± 8.45	14.06 ± 7.08
	cen2-deletion	3.41 ± 3.26	
type-C			18.43 ± 14.71

Figure 2. Structural Analysis of the Stably Maintained Dicentric Chromosomes

(A and B) Chromosomal DNAs were prepared from representative survivor strains obtained with strategy 2 (A1–A4, B1, B2, C1, and C2) and separated by PFGE. DNA samples were double digested with StuI and BglI before separation in (B). The indicated fragments (IC, IIC, and IIIC) contained the centromeres of chromosomes I, II, and III, respectively. The molecular weights of the IIIC fragment were different between the parental strains, probably because of a polymorphism in the number of repetitive pericentromeric elements.

(C) Southern blotting analysis of genomic DNAs from representative survivors after EcoRI and EcoRV double digestion. The positions of the probes are illustrated on the diagrams of centromeres 1 and 2. Arrows indicate the fragments missing in type-B survivors (B1 and B2); a large part of the central domain of centromere 1 appeared to have been deleted in these two survivors, whereas centromere 2 was intact.

(D) NcoI sites around the centromeres in representative survivor strains obtained with strategy 2 (A1–A4, B1–B3, C1, and C2) were mapped by partial digestion followed by PFGE and Southern blotting. Probes are indicated by horizontal bars.

(E) Proportions of the types of survivors. The means ± SD were calculated from at least three independent trials. A total of 77 and 126 survivor strains, which were isolated with strategies 1 and 2, respectively, were analyzed. The ratios of subtypes in type-A survivors were estimated by analyzing six and ten survivors in strategies 1 and 2, respectively (see also Figure S2).

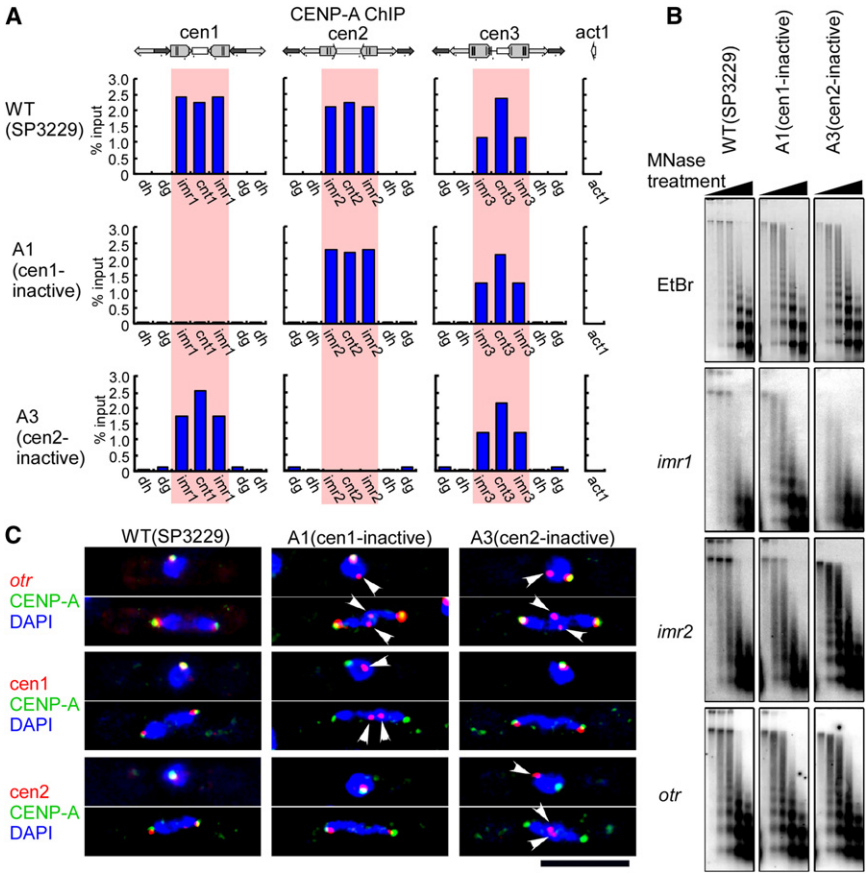


Figure 3. The Kinetochore Is Not Formed on the Inactivated Centromere in Type-A Survivors

(A) Chromatin DNAs on which CENP-A binds were isolated from cell extracts of WT (SP3229) and representative type-A survivors, A1 and A3 (SP3991 and SP3992), by ChIP, and quantified by real-time PCR. CENP-A accumulation was absent on the central domains (shown in orange) of centromere 1 in A1 cells and centromere 2 in A3 cells.

(B) Chromatin structures at the centromere regions were analyzed by micrococcal nuclease digestion followed by Southern blotting. In WT, a smear-like pattern was detected in the central domains (*imr1* and *imr2*), whereas a regularly spaced nucleosomal ladder was detected in the heterochromatin domain (*otr*). In type-A survivors, the smear-like pattern was missing on the central domain of the CENP-A-absent centromere.

(C) Centromere DNAs (red) were visualized by FISH using the *otr* probe hybridizing to all the centromeres, centromere 1-specific probe (cen1) and centromere 2-specific probe (cen2). CENP-A (green) was also visualized by immunofluorescence. Micrographs of a cell in interphase (upper) and mitosis (lower) are shown for each probe. Arrowheads indicate CENP-A-absent centromeres in type-A survivors. Scale bar represents 5 μm (see also Figure S3).

WT). In contrast, CENP-A accumulation on centromere 1 was lost in some type-A survivor strains (Figure 3A, strain A1), and that on centromere 2 was lost in other type-A survivors (Figure 3A, strain A3). In the central domains of the CENP-A-lacking centromere, canonical histone H3 appeared to replace CENP-A; chromatin immunoprecipitation (ChIP) with the anti-histone H3 antibody revealed that canonical histone H3 accumulated on the central domains of centromere 1 and 2 in strains A1 and A3, respectively, whereas it was excluded from these domains in all centromeres of the WT (Figure S3). These results suggested that the kinetochore was formed on only one of the centromeres on the dicentric chromosome in these survivors.

Micrococcal nuclease (MNase) digestion analysis indicated that the CENP-A-lacking centromere exhibited a ladder pattern similar to *otr*, instead of the smear-like pattern observed in the CENP-A-containing centromeres (Figure 3B). Because a smear-like structure corresponds to the active centromere [19], the disappearance of the smear-like nucleosomal structure in the CENP-A-lacking centromere may reflect loss of centromeric activity.

To further confirm whether the CENP-A-lacking centromere had lost its function, we examined the mitotic behavior of the centromeres on the dicentric chromosome. Figure 3C shows the results of fluorescence in situ hybridization (FISH) analyses using probes specific for centromere 1 and centromere 2 and an *otr* probe hybridizing to all of the centromeres. As reported previously [20], all of the centromeres were clustered in the vicinity of the spindle pole body in interphase and at the leading edges of dividing nuclei during mitosis in WT cells (Figure 3C, left column). In contrast, in type-A survivor strains, the CENP-A-lacking centromere was detached from the cluster of centromeres in interphase and segregated as part of the chromosome arm during mitosis. These results indicated that the CENP-A-lacking centromere no longer associated with the spindle apparatus, consistent with the loss of centromere activity as a platform for kinetochore assembly.

Taken together, these observations suggested that the kinetochore complex was formed on only one of the two centromeres on the dicentric chromosome in type-A survivors, although the DNA sequences of both centromeres were intact. Based on the results of these analyses, type-A survivor strains were classified into two subtypes: cen1-inactive and cen2-inactive, in which either centromere 1 or 2 did not function as a kinetochore formation site, respectively. Approximately half of the type-A survivors were cen1-inactive (Figure 2E). No type-A survivor strains were obtained in which both cen1-inactive and the cen2-inactive cells coexisted, indicating that the active and inactive states of each centromere were stable and did not switch reciprocally through generations.

Mutations Compromising the Kinetochore Promote Centromere Inactivation

We next examined how mutations compromising kinetochore assembly affect the frequency of stable dicentric chromosome maintenance by the survivors. The fission yeast kinetochore consists of two parts, i.e., the central core complex and flanking pericentric heterochromatin. First, the frequencies of survivors with the dicentric chromosome were measured in mutants defective in formation of the central core (Δ*ams2*, *mis6-302*, *mis12-537*, and *mis16-53*) (Figure 4A). The central core complex can be dissected into several distinct subcomplexes; Mis6 and Mis12 are integral components of the

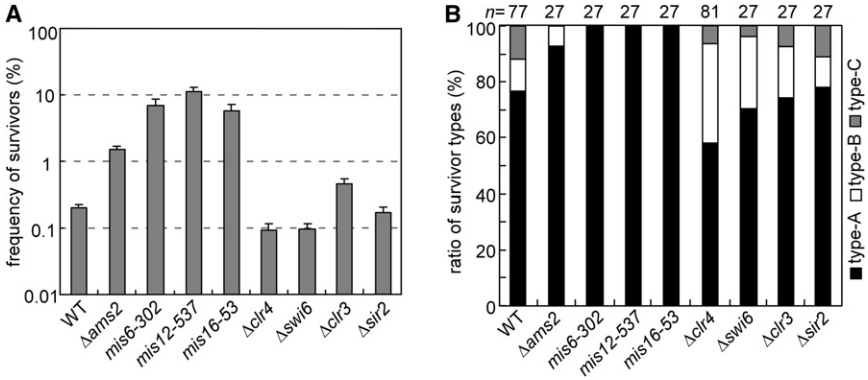


Figure 4. Mutations Compromising Kinetochore Formation, but Not Heterochromatin-Related Mutations, Increase the Frequency of Emergence of Type-A Survivors

(A) The dicentric fused chromosome was generated in the indicated mutant strains and the WT with strategy 1 and the frequency of emergence of survivors was estimated. Error bars represent the SD calculated from three independent experiments.

(B) The ratios of the types of survivor were estimated in the mutant strains. The indicated numbers (*n*) of survivors were isolated with strategy 1 in each mutant strain and categorized as described in the text (see also Figure S4).

Mis6/Sim4 subcomplex and KMN subcomplex, respectively, both of which are essential for equal segregation of sister chromatid in mitosis [19, 21]. On the other hand, Mis16 is homologous to human RbAp46/48, which is a component of chromatin assembly factor-1 (CAF-1) [22]. Mis16 forms a distinct complex with Mis18 essential for localization of CENP-A on the centromere throughout the cell cycle [23], whereas Ams2 is the GATA transcription factor required for S-phase-specific CENP-A recruitment [24]. Under semipermissive conditions, all of these mutations increased the frequency of the survivors by 10- to 50-fold in comparison to the WT, suggesting that partial dysfunction of the kinetochore may stabilize the dicentric chromosome. PFGE analysis revealed that the DNA of both centromeres on the dicentric chromosome was intact in nearly all the survivors. To confirm that these survivors were type-A, we performed ChIP analysis in randomly selected survivors (four survivors from *mis6* and two each from remaining mutants) using anti-CENP-A antibody (Figure S4A). In all the survivors tested, CENP-A bound to only one of the centromeres on the dicentric chromosome, indicating that virtually all the survivors obtained from these mutants were indeed type-A. Although each of the mutations examined here had a defect in a different aspect of kinetochore assembly and function, they all greatly facilitated centromere inactivation to stabilize the dicentric chromosome (Figure 4B). Therefore, we speculated that kinetochore disassembly is one of the key factors triggering centromere inactivation.

Heterochromatin Is Not a Prerequisite for Epigenetic Centromere Inactivation

Next, we examined whether pericentric heterochromatin was also involved in centromere inactivation. The frequencies of survivors with the dicentric chromosome were determined in heterochromatin-related mutants (Δ*clr4*, Δ*swi6*, Δ*clr3*, and Δ*sir2*) (Figure 4A). HP1-like Swi6 and Clr4, which is a histone H3 methyltransferase, are required for pericentric heterochromatin formation [25], whereas Clr3 and Sir2, which are class II and III histone deacetylases, respectively, are required for histone deacetylation in heterochromatic regions [26, 27]. In contrast to the mutations defective in the central core, these mutations did not significantly affect the total frequency of the survivors in comparison to the WT (Figure 4A). PFGE analysis indicated that the ratio of type-B survivors slightly increased at the expense of small decrease of the type-A in these mutants (Figure 4B). Loss of CENP-A binding on one of the centromeres of the dicentric chromosome was confirmed by ChIP analysis in randomly selected type-A survivors (Figure S4B). These results suggested that pericentric heterochromatin is not required for epigenetic centromere inactivation in type-A survivors, although it may prevent rearrangement of the centromere DNA generating the type-B survivors.

The Central Domain of the Inactivated Centromere Is Heterochromatinized

We then examined the distribution of heterochromatin around the inactivated centromere in the type-A survivors. For this purpose, ChIP analyses using antibodies against histone H3 dimethylated at lysine 9 (H3K9me2) and Swi6 were performed (Figures 5A and 5B), because methylated histone H3 is a hallmark of heterochromatin, and Swi6 binds specifically to methylated histone H3 [25]; in the WT centromere, H3K9me2 and Swi6 bound to the *otr* domain consisting of *dg* and *dh* repeats, but not to the central domain (*imr* and *cnt*). Surprisingly, in the inactivated centromere in the type-A survivors, these proteins were detected in the central domain, indicating that the CENP-A-lacking central domain of the inactivated centromere was heterochromatinized in the type-A survivors. Because neither H3K9me2 nor Swi6 was found in the central domain of the active centromeres (e.g., centromere 2 in the cen1-inactive A1 strain), heterochromatinization of the central domain was strongly correlated with inactivation of the centromere. Similarly, in the type-A survivors isolated from kinetochore-defective mutants (*mis6-302*, *mis12-537*, and *mis16-53*), the central domain was also heterochromatinized in the inactivated centromere, but not in the active centromere (Figure S5).

We noticed that a small but significant amount of H3K9me2 was detected at the active centromeres in the survivors from Δ*ams2* mutant; this was consistent with the previous report that canonical histone H3 was ectopically incorporated into the central domain in the Δ*ams2* mutant [24].

Histone H3 in the Inactivated Centromere Is Hypoacetylated

Histones are generally hypoacetylated in heterochromatin [25]. Because the central domain of the inactivated centromeres was heterochromatinized in the type-A survivors, we examined the status of histone acetylation at the centromere in these survivors. The amounts of acetylated histone H3 at lysine 14 (H3K14) and at lysine 9 (H3K9) were measured by ChIP analysis (Figure 6A); these two residues are deacetylated in the pericentric heterochromatin in WT cells [26, 27]. In the control type-A survivor strain A3, H3K14, and H3K9 in the central domain of the inactivated centromere (*cnt2*) were

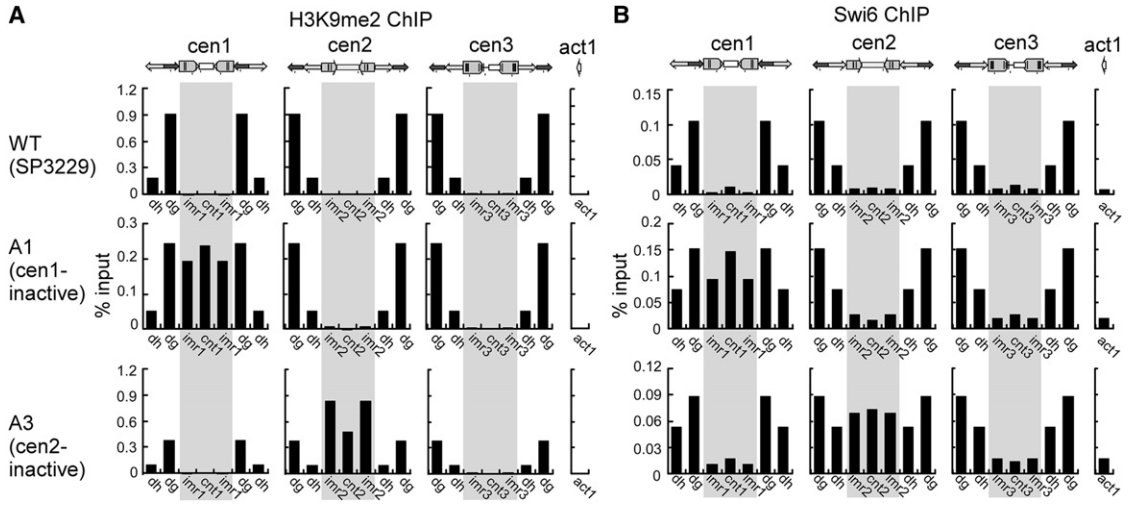


Figure 5. The Inactivated Centromere Is Heterochromatinized

Distributions of H3K9me2 (A) and Swi6 (B) at the centromeric regions were determined by ChIP and qRT-PCR. These heterochromatin components were found in the central domain (shown in gray) of the inactivated centromere in type-A survivor strains (see also Figure S5).

hypoacetylated to a degree similar to that in the pericentric heterochromatin domain (*dg*). Two histone deacetylases (HDAC), Clr3 and Sir2, presumably deacetylate histones in the inactivated centromere; the acetylation levels in H3K14 were elevated in the A3 derivative strain lacking the *clr3*$^+$ gene (Δ*clr3*), whereas those in both H3K14 and H3K9 were elevated in the A3 derivative strain lacking the *sir2*$^+$ gene (Δ*sir2*). This deacetylation of histones was important for heterochromatinization of the inactivated centromere, because the amount of Swi6 bound to the inactive centromere was reduced in the A3 derivative lacking either of the HDAC genes. Conversely, heterochromatinization was essential for histone deacetylation in the inactivated centromere, because both H3K9 and H3K14 in the *cnt2* domain as well as the *dg* domain were highly acetylated in the A3 derivative strains lacking the *clr4*$^+$ or the *swi6*$^+$ gene essential for heterochromatin formation (Figure 6A, Δ*clr4* and Δ*swi6*). On the other hand, exclusion of CENP-A from the inactive centromere did not require either heterochromatinization or deacetylation, because CENP-A did not accumulate on the inactive centromere in these A3 derivative strains.

It may be noteworthy that, in comparison to the A3 control, the level of H3K9me2 was markedly increased by deletion of the *clr3*$^+$ gene in the inactivated centromere, whereas it was unaffected in the pericentric heterochromatin domain (*dg*) (Figure 6A). Likewise, the H3K9me2 level in the inactive centromere was significantly reduced by *sir2*$^+$ gene deletion, whereas that in the *dg* domain was unchanged. These observations suggested that levels of histone modification may be regulated differently in the core domain of the inactivated centromere and the pericentric heterochromatin domain. These differences in histone modifications may be important for the suppression of centromere reactivation discussed below.

Heterochromatin Suppresses the Revival of Centromeric Activity in the Inactivated Centromere

Although heterochromatin was dispensable for centromere inactivation observed in the type-A survivors, it covered the central domain of the inactivated centromere and was essential for histone deacetylation. To investigate the role of heterochromatin in centromere inactivation, we examined whether it may prevent reactivation of the inactivated centromere. In this experiment, we tested whether the inactivated centromere would be reactivated following deletion of the other active centromere from the dicentric chromosome. By Cre-*loxP* site-directed recombination [3], active centromere 1 that was bracketed by *loxP* sites was excised from the dicentric chromosome of the cen2-inactive type-A strain A3. Although most cells did not proliferate, 0.07% of cells survived after induction of Cre recombinase (Figure 6B). ChIP analysis with anti-CENP-A antibody indicated that centromere 2 was not reactivated in any of the ten survivors tested; instead, a neocentromere appeared close to the chromosomal ends in eight survivors and somewhere else in the remaining two (Figure 6C, A3 control). Therefore, little reactivation of the inactivated centromere is thought to occur even when the active centromere has been eliminated. Surprisingly, when the experiment was performed using the A3 derivative strains lacking one of the HDAC genes (Δ*clr3* and Δ*sir2*), the inactivated centromere was reactivated in eight of ten survivors tested (Figure 6C). Because frequencies of total survivors after removal of the active centromere in these A3 derivative mutants were comparable to that in the control (Figure 6B, 0.06% and 0.04% in Δ*clr3* and Δ*sir2*, respectively), these results indicated that deleting either of the HDAC genes strongly increased the probability of reactivation of the once-inactivated centromere. Similarly, although the frequencies of total survivors were decreased to 0.02% and 0.01% in Δ*clr4* and Δ*swi6* mutants, respectively (Figure 6B), reactivation of the inactivated centromere occurred in one-third of the survivors in these mutants (Figure 6C). These results also suggest that the probability of centromere reactivation was elevated by deleting genes required for heterochromatin formation, although it is also possible that these results may simply reflect the reduction of neocentromere formation in these mutants as reported previously [3]. Taken together, these results suggest that histone deacetylation, and presumably heterochromatinization, may prevent reactivation of the inactivated centromere, which retains the potential to form a functional kinetochore.

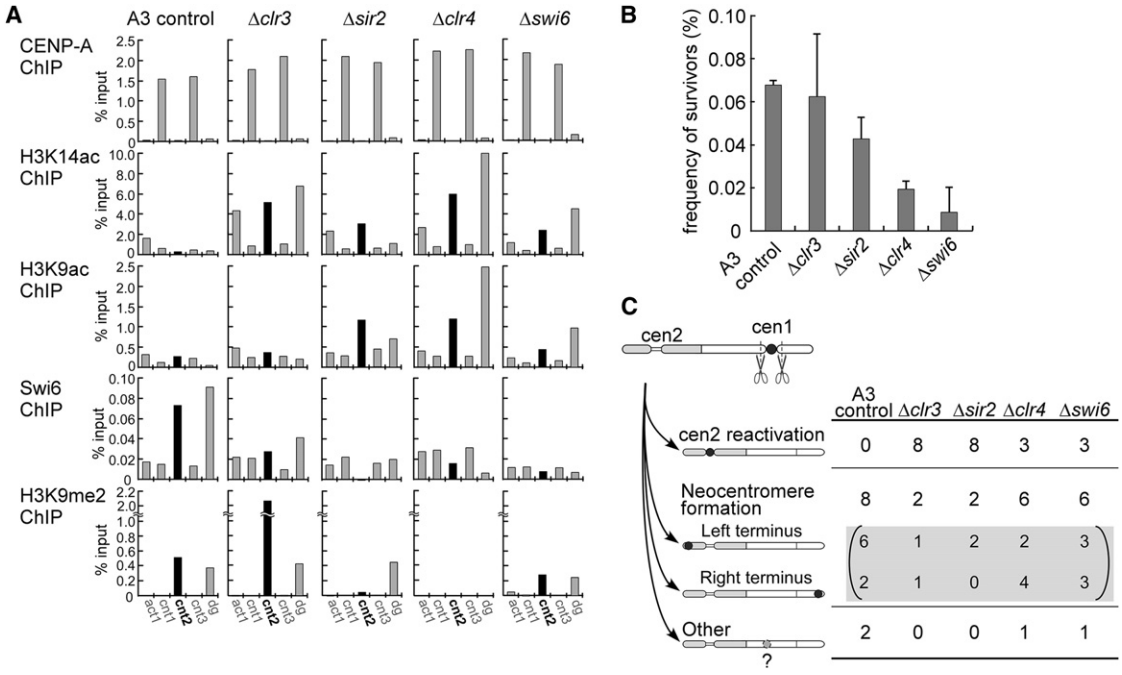


Figure 6. The Inactivated Centromere Is Frequently Reactivated in Heterochromatin- and HDAC-Deficient Mutants

(A) Distributions of CENP-A, H3K14ac, H3K9ac, Swi6, and H3K9me2 at the central domains and pericentric repeat (*dg*) were determined by ChIP in the type-A survivor A3 (A3 control, SP4238) and its derivatives in which the *clr3*$^+$ (Δ*clr3*), *sir2*$^+$ (Δ*sir2*), *clr4*$^+$ (Δ*clr4*), or *swi6*$^+$ (Δ*swi6*) gene was disrupted. In these strains, the inactivated centromere was centromere 2, the central domain of which (*cnt2*) is indicated in black bold.

(B and C) The active centromere 1 was excised from the fused chromosome in cen2-inactive A3 cells (A3 control, SP4238) and in A3 derivative cells lacking the *clr3*$^+$ (Δ*clr3*), *sir2*$^+$ (Δ*sir2*), *clr4*$^+$ (Δ*clr4*), or *swi6*$^+$ (Δ*clr6*) gene. The frequency of total survivors after removal of the active centromere was estimated in the parental A3 control strain and its derivatives (B). Error bars represent the SD calculated from three independent experiments. Ten strains surviving removal of centromere 1 were isolated in each genetic background, and the position of the kinetochore on the fused chromosome was determined by ChIP with anti-CENP-A antibody (C). The numbers of survivors in which the kinetochore formed at the indicated position (filled circles) are shown in the table. "Other" means that kinetochore formation was not detected on either the centromere 2 or neocentromere loci.

It should be noted that reactivation of the inactivated centromere as well as neocentromere formation became prominent only when the active centromere was disrupted; otherwise, these events would generate a dicentric chromosome, and cells harboring a dicentric chromosome would be eliminated promptly under nonselective conditions.

Discussion

In this study, we developed model systems for formation of a dicentric chromosome by fusing two chromosomes in fission yeast and examined the physiological consequences of the presence of multiple centromeres on a single chromosome. The majority of the fission yeast cells harboring a dicentric chromosome were arrested in interphase, although a small portion of the cells escaped from the arrest presumably by converting the dicentric chromosome into a monocentric chromosome or breaking it into two chromosomes. In contrast, in other organisms such as maize, dicentric chromosomes do not appear to cause cell-cycle arrest, but result in mitotic abnormalities, such as breakage and loss of chromosomes due to disorganized spindle-chromosome interaction [1, 28–30]. The reason for this difference among organisms remains unclear. Because dicentric chromosome-induced cell-cycle arrest requires the DNA damage and replication checkpoint genes in fission yeast, DNA ends generated by mitotic breakage of the dicentric chromosome may be hardly healed and thus permanently activate the DNA damage checkpoint restraining mitotic onset in some organisms. The other possibility is that some organisms, including fission yeast, may have developed a checkpoint-like mechanism that inhibits entry into mitosis when more than two centromeres exist on a single chromosome. If such cell-cycle regulation mechanisms do exist, epigenetic centromere inactivation may be promoted during cell-cycle arrest in interphase.

Detailed PFGE analyses strongly suggested that, in nearly 70% of the survivors with the dicentric fused chromosome, one of the centromeres was epigenetically inactivated without accompanying alterations in the DNA sequence. The epigenetically inactivated centromere presumably retained the potential for kinetochore formation, because it could regain the function at least in HDAC- and heterochromatin-deficient mutant strains. Such epigenetically inactivated centromeres, which retain the potential to be reactivated, have been reported in humans, plants, and *Drosophila* [31–33]. Epigenetic inactivation of the centromere is, therefore, a phenomenon commonly observed in eukaryotes harboring regional centromeres; this phenomenon may play a crucial role in not only stabilizing the dicentric chromosome but also limiting the number of centromeres per chromosome. Survivors harboring the dicentric chromosome occurred at a higher frequency in mutants defective in kinetochore formation than in the WT controls (Figure 4A). Doheny et al. reported previously that two mutations in the kinetochore components in budding yeast, *ctf13-30* and *ndc10/ctf14-42*, stabilized a dicentric chromosome [34]. Although they speculated that the dicentric

chromosome would be stabilized by weakened attachment of the mutant kinetochore to the mitotic spindle leading to detachment of spindle microtubules before chromosomal breakage, it appears unlikely to occur in fission yeast. Our PFGE and ChIP analyses indicated that the frequency of type-A survivors, in which the dicentric chromosome was stabilized by epigenetic centromere inactivation, was greatly increased in *mis6*, *mis12*, *mis16*, and Δ*ams2* mutants, in which the kinetochore tends to disassemble. Hence, we assume that epigenetic centromere inactivation is triggered by kinetochore disassembly, which is followed by heterochromatinization.

As the frequencies of type-A survivors in heterochromatin- and HDAC-deficient mutants were comparable to that in the WT control, heterochromatinization is not a prerequisite for epigenetic centromere inactivation. Rather, heterochromatinization following centromere inactivation suppresses the revival of centromere activity. Suppression of centromere revival by heterochromatin is consistent with a previous report that experimentally induced heterochromatin within the centromere is incompatible with kinetochore activity [35]. This raises the question of how heterochromatin suppresses revival of the inactivated centromere. We presume that alterations in histone modification accompanied with heterochromatinization, such as acetylation, prevent reactivation. Consistent with this suggestion, the inactivated centromere was frequently reactivated in mutants lacking Clr3 or Sir2, which are involved in deacetylation of histones in the inactivated centromere. Previous reports suggested that hMis18 and RbAp46/48 (humans Mis16 homolog), which are required for deposition of de novo synthesized CENP-A, regulate the acetylation status of centromeric histones in humans [36]. Therefore, changes in acetylation status of histones at the inactivated centromere may inhibit incorporation of CENP-A.

Systematic comparison of chromosome structure among eukaryotic species indicated that chromosome fusion has occurred frequently in the history of chromosome evolution to generate dicentric chromosomes, and either centromere was inactivated by as yet unknown mechanisms [37, 38]. Therefore, this study provided new insights into the mechanism of chromosome evolution.

Experimental Procedures

General Techniques, DNA Manipulation, Microscopy, and Centromere Disruption

General fission yeast methods and media were described previously [39]. YES was used as rich medium, and EMM2 with appropriate supplements was used as minimal medium. PFGE was performed as described [3]. ChIP assays were performed as described [3], using anti-H3K9me2 (Abcam), anti-Swi6 (Cosmo Bio), anti-H3K9ac (Upstate), anti-H3K14ac (Upstate), anti-histone H3 (Abcam), or anti-CENP-A (Cnp1) [40] antibodies. Immunofluorescence staining and FISH were carried out as described previously [41]. Centromere 1 was disrupted as described previously [3], with some modifications.

Generation of a Dicentric Chromosome by Chromosome Fusion

Strategy 1

A dicentric fused chromosome was produced by Cre-mediated homologous recombination at *loxP* sites introduced at the ends of chromosomes I and II in SP4064. Cells maintaining the fused dicentric chromosome were selected on solid YES medium containing 50 mg/L G418 and 1 g/L 5-FOA (Wako Pure Chemicals).

Strategy 2

A dicentric fused chromosome was generated by meiotic crossover between the SP3970 strain and the WT strain (SP3229). SP3970 contains a fused chromosome composed of chromosome II and centromere-disrupted chromosome I. Progeny harboring the dicentric chromosome were selected on solid EMM2 medium containing 1 g/L 5-FOA and 70 mg/L uracil but lacking leucine.

See Supplemental Experimental Procedures for details.

Supplemental Information

Supplemental Information includes five figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2012.02.062.

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